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A 2D NMR study of the internal flexibility of the antifungal peptide stendomycin

J. P. Simorre [1], D. Genest [1], A. Caille [1], and M. Ptak [2]

[1] CBM-CNRS 1A, avenue de la recherche scientifique F-45071 Orléans, Cedex 02, France
[2] Université d'Orléans, UFR Sciences, B.P. 6759, F-45067 Orléans Cedex 02, France

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Abstract. A 2-D ^1H NMR study (NOESY, COSY, HOHAHA and ROESY experiments) of the antifungal peptide stendomycin is presented. The variation of the NOESY cross peak intensities is measured as a function of temperature in order to discriminate between constant and fluctuating interproton distances. It is shown that among 71 NOESY cross peaks, only 12 correspond to well defined interproton distances and their correlation time is determined. The other cross peaks cannot be translated accurately in terms of distances owing to internal molecular motions. $\{^1H\}-^{13}C$ nOe measurements confirm the internal mobility of the molecule. Finally a flexibility map of stendomycin can be established.

Key words: Molecular dynamics – 2-D NMR – Correlation times – Structure determination – Antifungal

Introduction

Stendomycin is an antifungal tetradecapeptide isolated by Thomson and Hughes from cultures of *Streptomyces endus* at the Lilly Research Laboratory (Thomson and Hughes 1963). According to Bodansky et al. (1969), the major component contains two unusual residues: stendomycidin and dehydrobutyrene, and is composed of a lactone ring involving seven residues, a linear side chain involving seven other residues and a terminal fatty acid.

$C_{13}H_{27}$CO–Pro–NMe-L-Thr–Gly–D-Val–D-alle–D-Ala–
–But–D-aThr–D-Val–L-Val
O D-aThr
L-Ste–D-alle–L-Ser

Stendomycin is soluble in most common organic solvents and slightly soluble in water. Urry and Ruiter (1970) have shown that its conformation is solvent-dependent but rather insensitive to temperature and Pitner and Urry (1972) have proposed a model of one possible conformation in trifluoroethanol based on classical one-dimensional (1D) ^1H NMR studies. The biological mechanism of action of stendomycin has not yet been elucidated.

Current two-dimensional (2D) NMR techniques have proved to be powerful tools for the conformational analysis of peptides. The methods are based on the knowledge of a number of interproton distances deduced from the values of nuclear Overhauser enhancements (nOe) (Wüthrich et al. 1982). In the last few years, we have used such techniques to investigate the conformation in solution of several antifungal lipopeptides of the iturin family constituted by a cyclic heptapeptide and a fatty acid side chain (Marion et al. 1986; Genest et al. 1987; Marion et al. 1987).

Two difficulties are often encountered in ^1H NMR studies of such medium size molecules: *a)* for measurement frequencies ranging from 300 to 500 MHz and at room temperature in normal solvents the nOe intensities can be vanishingly small and can even be equal to zero when the rotational correlation time, τ_c, is such that $\omega\tau_c \simeq 1$. *b)* large internal motions cause complicated averaging effects which make the interpretation of nOes very difficult.

Different methods, including ROESY measurements (Bothner-By et al. 1984; Bax and Davis 1984), have been proposed to overcome the first difficulty. It is considerably more difficult to analyze the molecular dynamics, especially when the correlation time τ_c becomes longer than $10^{-9}-10^{-8}$ s, a range which is rather long for such calculations. For cyclic peptides such as those we consider here, some parts of the molecules can be expected to be rigid enough to have fairly constant interproton distances whereas other parts will be flexible with fluctuating interproton distances. Therefore the first step in a conformational analysis might be to discriminate between these proton pairs in order to start further modeling.

Offprint requests to: J. P. Simorre

Variations of the ^{13}C relaxation parameters (T_1, T_2 or nOe) along a peptide chain give a picture of its internal dynamics which can be interpreted in terms of motions of the C−H vectors (Schaefer 1973), but do not give direct information on H−H vector dynamics.

Fluctuations of the interproton distances can be detected by analysing temperature effects and we have recently proposed the use of such an experimental approach to investigate internal motions (Genest and Simorre 1990).

Stendomycin, which contains a cyclic part and a linear part seems to be especially well suited to such studies and it should be a reference for our further studies of antibiotic peptides and proteins.

In the present work, we present the experimental results of a ^1H and ^{13}C NMR study which enable us to give a qualitative picture of the flexibility of stendomycin. These results provide the basis for rationalizing the use of molecular mechanics methods and then of molecular dynamics methods to elaborate models of conformations of partly flexible molecules.

Materials and methods

1. Biochemicals

Stendomycin was a gift from Pr. G. Michel, Université C. Bernard, Lyon.

2. NMR

All NMR measurements were performed on a Bruker AM300 WB spectrometer equipped with an Aspect 3000 computer. 6.3 mg of stendomycin, dissolved in 500 µl d_6-DMSO ($7.8\ 10^{-3}$ M) was placed in a 5 mm diameter NMR tube for ^1H NMR experiments. The sample was degassed and sealed under argon atmosphere. For ^{13}C experiments 66 mg of stendomycin was dissolved in 2 ml of d_6-DMSO ($20 \cdot 10^{-3}$ M) and placed in a 10 mm diameter NMR tube. The assignment of the ^1H resonance lines was made according to routine two-dimensional spectra: DQF-COSY (Piantini et al. 1982) and HOHAHA with MLEV17 mixing (Davis and Bax 1985) for intraresidue proton connectivities, and NOESY (Macura and Ernst 1980) and ROESY (Bothner-By et al. 1984) for the sequential assignment and for determination of distances. The pulse sequence for 2-D ROESY included a train of pulses to suppress J cross peaks (Kessler et al. 1987) and supplementary pulses for elimination of offset effects (Griesinger and Ernst 1987).

All spectra were acquired in the phase sensitive mode (TPPI) (Marion and Wüthrich 1983) with 256 tl increments of 4096 data, and Fourier transformed with zero-filling in the t_1 dimension.

The DQF-COSY and NOESY experiments were recorded at 295 K, 301 K, 307 K and 313 K. The mixing times (τ_m) in NOESY were 80 ms, 140 ms, 200 ms and 300 ms at 295 K and 140 ms at the other temperatures. ROESY was done with $\tau_m = 140$ ms at 295 K and HOHAHA with $\tau_m = 52$ ms and 73 ms at 301 K.

The assignment of the ^{13}C spectrum was based on a heteronuclear $^{13}C-^1H$ COSY experiment in the magnitude mode (Bax and Morris 1981) at 295 K. A 512 · 4096 matrix was acquired, and each FID required 896 scans. After one zero filling in the t_1 dimension and Fourier transformation with apodization functions (Gaussian in t_2 dimension and sine square in t_1 dimension), the ^{13}C resonances were identified from their connectivities with the previously assigned ^1H resonances.

The nuclear Overhauser enhancement factor for the different ^{13}C nuclei is given by the ratio I_0/I, where I_0 and I are the integrals of the ^{13}C lines of the ^1H non-decoupled and ^1H decoupled 1D spectra respectively (Doddrel et al. 1972). The correlation times of the $^{13}C-^1H$ pair are deduced from the following relationship (Schaefer 1973):

$$\mathrm{NOE} = 1 + \frac{g_H}{g_C}\left(\frac{6J(\omega_H+\omega_C) - J(\omega_H-\omega_C)}{J(\omega_H-\omega_C) + 3J(\omega_C) + 6J(\omega_H+\omega_C)}\right)$$

with

$$J(\omega) = \frac{\tau_{CH}}{1+\omega^2\tau_{CH}^2}$$

I_0: ^{13}C magnetization with proton irradiation; I: ^{13}C magnetization without proton irradiation; g_H: gyromagnetic ratio of ^1H; g_c: gyromagnetic ratio of ^{13}C; ω_H: Larmor angular velocity of ^1H; ω_C: Larmor angular velocity of ^{13}C.

3. Analysis of NOESY spectra

It is well known that the intensity a_{ij} of a cross peak between two resonance lines in NOESY depends on the dipolar cross relaxation rate between the corresponding protons. As the mixing time τm approaches zero, the cross peak intensity a_{ij} becomes proportional to the longitudinal cross relaxation rate σ_{ij} (Macura and Ernst 1980). In most works published to date, σ_{ij} is assumed to be given by:

$$\sigma_{ij} = \frac{q_{ij}}{r_{ij}^6} \tag{1}$$

where r_{ij} is the distance between both protons and

$$q_{ij} = \frac{g^4\hbar^2}{10}\frac{(\mu_0)^2}{(4\pi)^2}\tau_{cij}\left(\frac{6}{1+4\omega^2\tau_{cij}^2} - 1\right)$$

In this last expression g is the gyromagnetic ratio of ^1H, $\hbar$ the Planck's constant divided by 2π, μ_0 the permeability in vaccuo, τ_{cij} the effective correlation time of the vector joining protons i and j, and ω is the Larmor angular velocity of the proton.

If one interproton distance of the molecule under investigation is known, it can be taken as a reference and all other interproton distances may be deduced from the relation:

$$r_{ij} = r_{ref}\left(\frac{a_{ref}}{a_{ij}}\right)^{1/6} \tag{2}$$

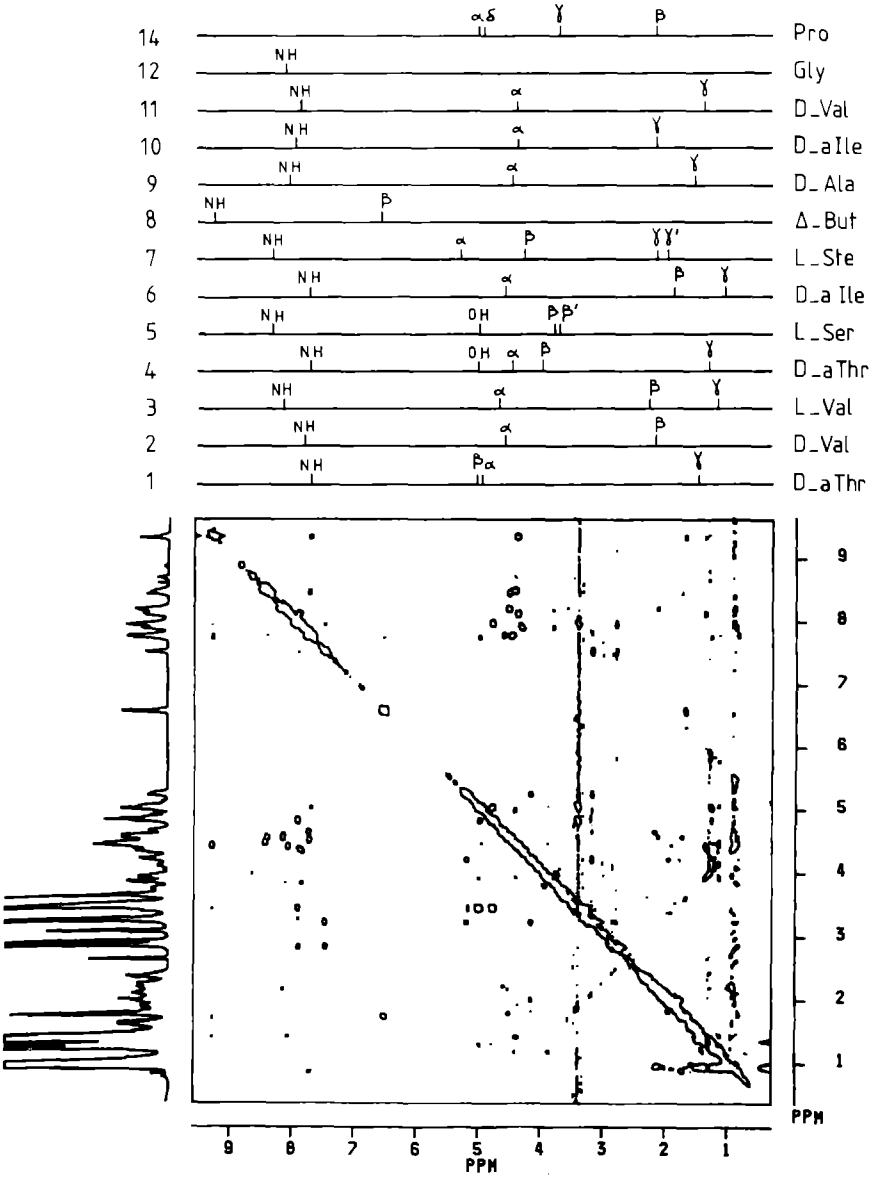


Fig. 1. NOESY map and ^{1}H assignment of stendomycin obtained with a mixing time of 140 ms at 295 K

Equation (2) is valid only if r_{ij} is not time-dependent, and if the effective correlation time is the same for each inter-proton vector of the molecule. We now briefly recall the method we proposed to check if the distance is constant and to determine the corresponding relaxation time (Genest and Simorre 1990). It rests on the variation of the NOESY cross peak intensities with η/T (η = solvent viscosity, T = absolute temperature). If r is constant and if the angular autocorrelation function G of r_{ij} can be approximated by a single exponential, then (1) is correct. Since the correlation time is proportional to η/T, $\tau_{cij} = k_{ij}\,\eta/T$. If one value η_0/T_0 is taken as reference the theoretical ratio

$$R_T(\eta/T) = \frac{\sigma_{ij}(\eta/T)}{\sigma_{ij}(\eta_0/T_0)} = \frac{\eta/T}{\eta_0/T_0} \cdot \left(\left(\frac{6}{1+4\omega^2 k_{ij}^2 (\eta/T)^2} - 1\right) \Big/ \left(\frac{6}{1+4\omega^2 k_{ij}^2 (\eta_0/T_0)^2} - 1\right)\right)$$

can be computed as a function of η/T for different values of k_{ij}. $R_T(\eta/T)$ does not depend on r_{ij}. Experimentally, NOESY spectra are measured at different values of η/T and one of them is chosen as reference (η_0/T_0). For each pair of protons the experimental ratio $R_E^{ij}(\eta/T) = (a_{ij}(\eta/T)/a_{ij}(\eta_0/T_0))$ is reported as a function of η/T. If the curve $R_E^{ij}(\eta/T)$ fits $R_T(\eta/T)$ with a particular value of k_{ij}, this means that the assumptions used to calculate R_T (r_{ij} is constant and G is monoexponential) are satisfied for this pair of protons in the investigated η/T range. The value of k_{ij} gives the corresponding proportionality constant between the correlation time and η/T. This allows one to determine r_{ij} by the relation:

$$\frac{a_{ij}}{a_{ref}} = \frac{r_{ref}^6}{r_{ij}^6}\,\frac{k_{ij}}{k_{ref}} \cdot \left(\left(\frac{6}{1+4\omega^2 k_{ij}^2 (\eta/T)^2} - 1\right) \Big/ \left(\frac{6}{1+4\omega^2 k_{ref}^2 (\eta_0/T_0)} - 1\right)\right)$$

On the other hand, if the experimental curve does not fit the theoretical one, this means that either r_{ij} does not have a single correlation time or that the interproton distance is not constant. As it is not possible to distinguish between these hypotheses the nOe intensity cannot be used to calculate distances reliably.

In our work the value of η_0 is 2.1 Cp (Marsden 1963) which corresponds to the viscosity of DMSO at $T_0 =$ 295 K. The reference distance is the average distance between H_β and the three methyl H of the dehydrobutyrene residue. This distance is 0.268 nm. We point out that the rotation of the CH_3 group about the $C_\beta - C$ bond has only a small influence on the nOe intensity, for this geometrical configuration (Tropp 1980).

Results

Figure 1 shows the assignment of the ^1H spectrum and an example of a NOESY map is given. Figure 2 shows the variation of some NOESY cross peak intensities measured at 295 K for different values of the mixing time τ_m. It can be seen that the intensities are nearly proportional to τ_m in the range $0 \leq \tau_m < 140$ ms. This has been observed for all cross peaks which could be quantified. Consequently all the NOESY experiments as a function of the temperature have been performed with $\tau_m = 140$ ms. This minimizes spin diffusion while the cross peak intensities are sufficiently strong to be measured. The chemical shifts and the nOe connectivities network do not reveal a conformational change between 295 and 313 K. This is in agreement with the work of Urry and Ruiter (1970).

Examples of NOESY cross peak intensities as a function of temperature are shown in Fig. 3. It is found that the intensities do not change in the same way with temperature. We have analyzed this variation as described in the Materials and Methods section. Figure 4 shows the experimental curves $R_E(\eta/T)$ as a function of η/T for a

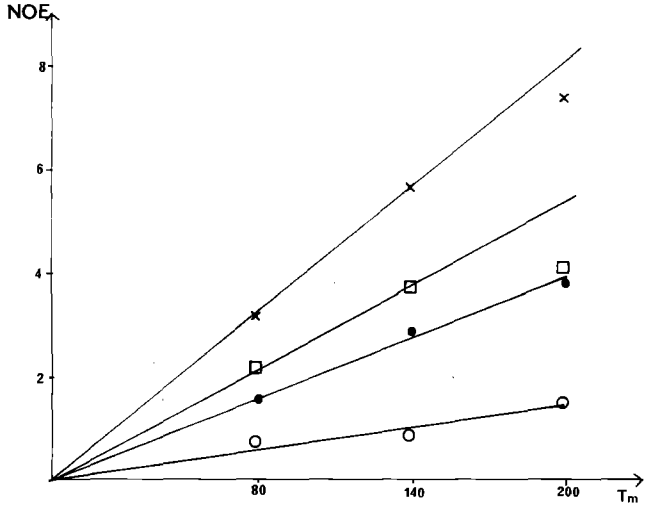


Fig. 2. Examples of NOESY cross peak intensities at 295 K as a function of mixing time. × = NH(9)−C_αH(9); □ = NH(7)−C_αH(2); ● = NH(8)−C_αH(7); ○ = NH(6)−C_αH(6)

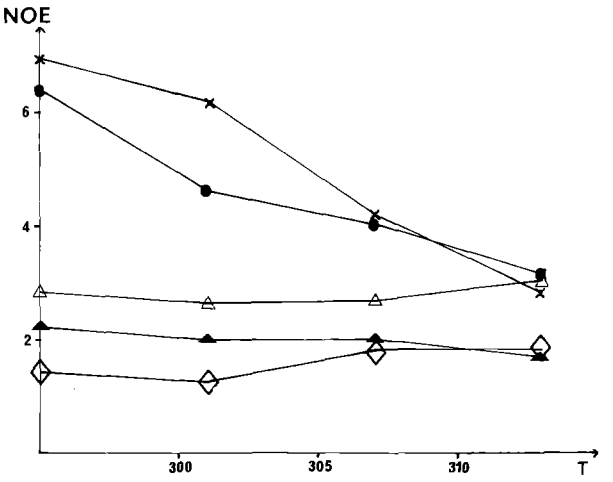
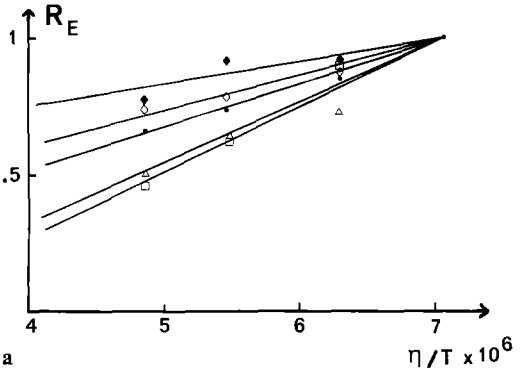


Fig. 3. Examples of NOESY cross peak intensities (mixing time = 140 ms) as a function of temperature. × = NH(8)−C_αH(9); ● = NH(3)−C_αH(3); △ = NH(4)−C_αH(3); ▲ = NH(8)−NH(1); ◇ = NH(2)−C_βH(1)



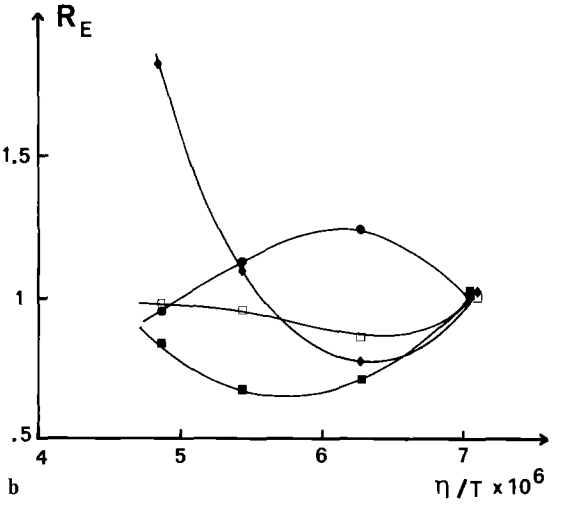


Fig. 4a, b. Ratio R_E as a function of η/T (in m^{-1} kg s^{-1} K^{-1}) for a few proton pairs (see text for definition of R_E). **a** ◆ = NH(8)−NH(1); ◇ = NH(11)−C_αH(11); ● = C_βH(8)−C_γH(8); △ = NH(3)−C_αH(3); □ = NH(8)−C_αH(9). The full lines correspond to the best R_T (see text) curves obtained by a linear least square fitting. **b** ■ = NH(7)−C_αH(2); □ = NH(2)−C_αH(1); ● = NH(4)−C_αH(4); ◆ = NH(8)−NH(2)

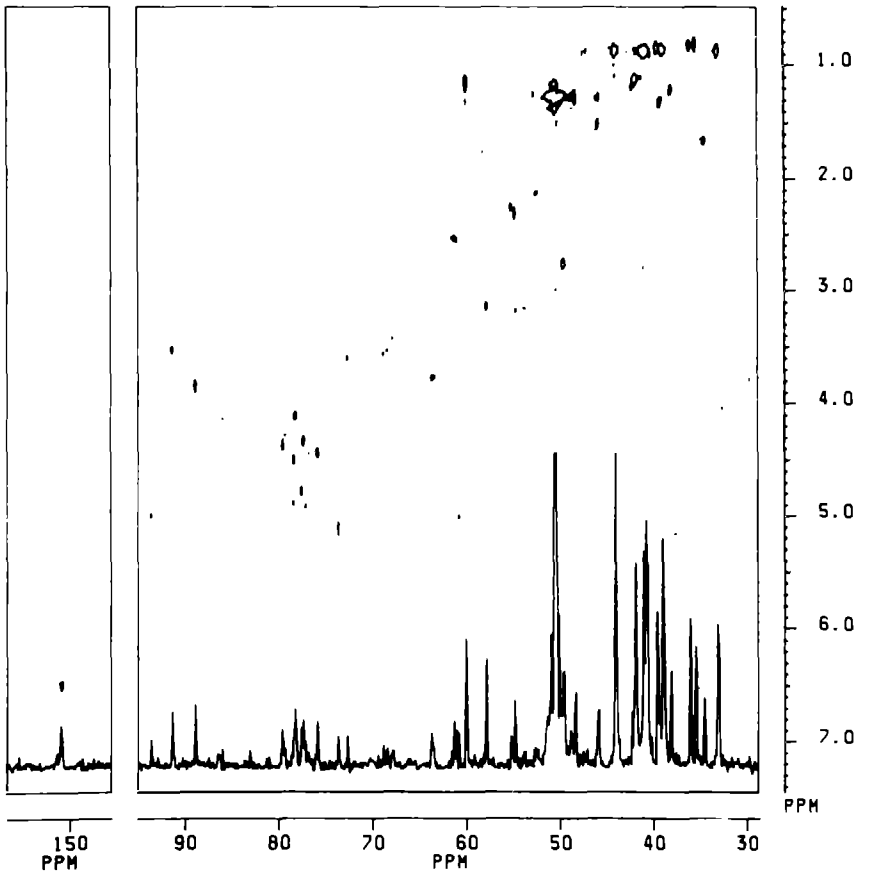


Fig. 5. Heteronuclear $^{13}C-^{1}H$ COSY map at 295 K

Table 1. Constant interproton distances (in Å) deduced from NOESY and ROESY experiments and corresponding correlation times (in ns) at 295 K

Protons	NOESY distances	ROESY distances	Correlation times
NH(1)–C_βH(1)	2.9	2.9	>3
NH(1)–NH(8)	2.9	2.1	>3
C_αH(2)–C_βH(2)	2.6	2.5	1.1
NH(3)–C_αH(3)	2.1	2.2	1.4
NH(5)–C_αH(5)	2.0	2.1	1
C_βH(7)–C_γH(7)	3.1		2.1
C_βH(7)–C_δH(7)	3.2		2.1
C^βH(8)–C_γH(8)	2.7	2.7	2.1
NH(8)–C_αH(9)	2.0	2.1	1.3
NH(9)–C_αH(9)	2.7	2.6	>3
NH(11)–C_αH(11)	2.6	2.5	>3
NH(11)–C_αH(12)	3.0	2.6	2.1

Table 2. NOe factor for different ^{13}C nuclei (I_0/I) and correlation times of the corresponding C–H vectors at 295 K

Carbons	I_0/I	Correlation times
C_βH(1)	1.38	1.8
C_αH(2)	1.38	1.8
C_αH(3)	1.56	1.1
C_αH(5)	1.49	1.4
C_αH(6)	1.51	1.3
C_αH(7)	1.25	3.2
C_βH(7)	1.56	1.1
C_βH(8)	1.0	>3
C_αH(10)	1.68	0.9
C_αH(12)	1.38	1.8

few pairs of protons i and j. They are compared to the theoretical ones, calculated with different values of k_{ij}. One can observe that for some pairs of protons $R_E(\eta/T)$ fits $R_T(\eta/T)$ (Fig. 4a) whereas for others it does not (Fig. 4b). We have detected 71 NOESY connectivities, but only 23 were sufficiently intense and well resolved to be quantified over the whole range of temperature and 12 of them follow the theoretical law. The values of the corresponding interproton correlation times at 295 K are given in Table 1, as well as the corresponding interproton distances. In the same table are reported for comparison the distances deduced from a ROESY experiment recorded at 295 K. The agreement of many of the distances obtained from NOESY or ROESY shows that artifacts caused by offsets and spurious HOHAHA cross peaks are well suppressed in our ROESY experiment. Only one proton pair (NH_1-NH_8) exhibits a difference.

Figure 5 shows the heteronuclear $^{13}C-^{1}H$ COSY map. We were able to identify most of the α carbon nuclei as well as some β carbon nuclei. All the ^{13}C resonances cannot be identified owing to overlap in the ^{1}H spectrum and to the low sensitivity of this experiment. Among the

assigned ^{13}C resonances, ten are sufficiently resolved for measuring their nOe factor and the effective correlation time of the corresponding $^{13}C-{}^{1}H$ vector. These values are reported in Table 2.

Discussion and conclusion

In the present work we were concerned with the internal flexibility of the antifungal peptide stendomycin. This was monitored by the two-dimensional ^{1}H NOESY experiment, and the variation of the NOESY cross peaks with temperature. Such an approach implies that the measured cross peak intensities are proportional to the dipolar cross relaxation rate between two protons. We verified that within the range $0 \leq \tau_m \leq 140$ ms all the cross peak intensities are proportional to τ_m. This means that although some spin diffusion mechanism may exist in this range of τ_m it is certainly not very significant.

It was observed that the cross peak intensities are temperature-dependent. This variation is not identical for all pairs of protons which suggests heterogeneity in the internal dynamics of the molecule. We have interpreted this variation in terms of diffusional Brownian dynamics. In this theory the reorientational correlation time of the vector is proportional to η/T. This approach is justified because the observed correlation times are in the range 1–3 ns which correspond to motions exhibiting a stochastic character. Furthermore, the high frequency motions corresponding to vibrations of individual atoms have too small an amplitude and their contribution to NOESY cross peak intensities are too weak to be detected experimentally (Olejniczak et al. 1984). The motions responsible for an effective reorientational relaxation are the overall rotational motion of the whole molecule and intramolecular motions of groups of atoms (McCammon and Harvey 1987). In both cases, the dynamics of the system may be described by the Langevin equation, where the frictional coefficient, in the case of this medium sized peptide, is dominated by the influence of the solvent viscosity.

The proportionality between the correlation time and η/T has been established theoretically only in the case of rigid molecules, and has not been rigorously proved in the case of flexible molecules. However it has often been experimentally observed (mainly by transient fluorescence anisotropy techniques) that optical transition moment vectors for a flexible macromolecule exhibit correlation times which are proportional to η/T (Genest and Wahl 1978; Claesens and Rigler 1986). This justifies the approach used in this work, which was extensively discussed in a previous paper (Genest and Simorre 1990).

Our analysis shows that among the 23 cross peaks we were able to quantify for all investigated temperatures, only 12 can be translated in terms of distances with certainty. The corresponding pairs of protons belong to a rigid part of the molecule. Their correlation times and their distances can be determined. For these distances the ROESY experiment gives practically the same values (Fig. 5). In contrast, the other quantified cross peaks tell us either that the corresponding protons fluctuate about their equilibrium position or are subject to a local conformational change. An alternative explanation is that the angular autocorrelation function differs greatly from a simple exponential. This reflects a fast local motion although the interproton distance could be constant. In any case these protons belong to a flexible part of stendomycin. These observations allowed us to establish a flexibility map for the peptide (Fig. 6). Examination of this map shows that the junction between the ring and the tail (Residues 8 and 1) is rather rigid. This rigidity could be due to the possibility of hydrogen bonding between residues 1, 8 and 9. Furthermore, the existence of NOESY cross peaks between the NH of residue 8 and the NH's of residues 2 and 7 strongly suggests a folding of the linear part of the molecule over the ring, although the distances between these residues show considerable fluctuation.

The method used to determine the interproton correlation times is accurate up to about 3 ns for a Larmor frequency of 300 MHz (Genest and Simorre 1990). We found that 4 of the interproton vectors have a correlation time of about 3–4 ns. We point out that monolayer experiments at the air-water interface give a radius of about 1.2 nm for the molecule when considered as a sphere (Maget-Dana, personal communication). This value leads to a global rotational correlation time of about 3.5 ns at

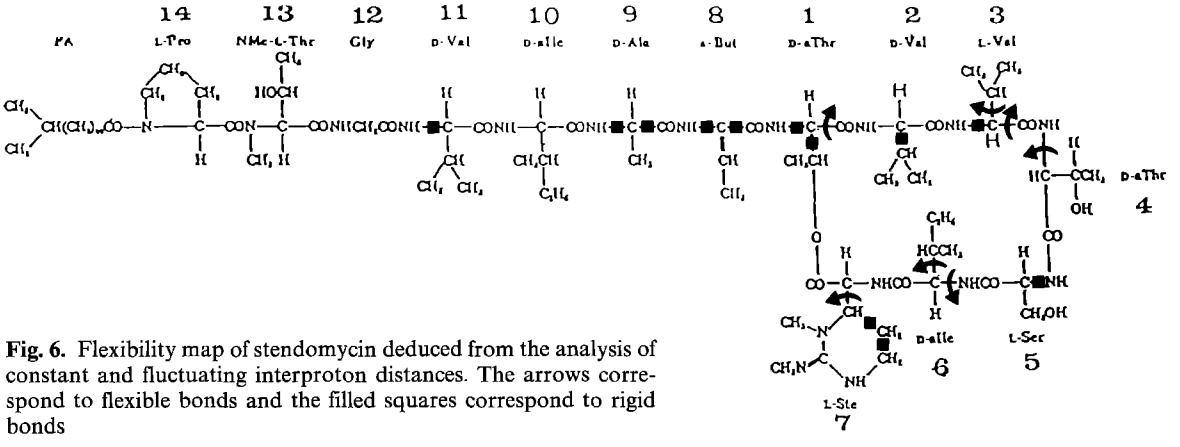


Fig. 6. Flexibility map of stendomycin deduced from the analysis of constant and fluctuating interproton distances. The arrows correspond to flexible bonds and the filled squares correspond to rigid bonds

295 K which is similar to the highest value determined in this work. The radius of 1.2 nm is consistent with the value observed from molecular design for the monomer.

For comparison we have determined the correlation times for a few $C_\alpha H$ and $C_\beta H$ vectors, by measuring the nOe in ^{13}C experiments. It was not possible to obtain this information from T_1 or T_2 of ^{13}C nuclei because the theoretical values are insensitive to τ_C for the investigated range of relaxation times. This is the reason why we measured the nOe factor of the ^{13}C nucleus. The range of accuracy in the determination of τ_C for the ^{13}C Larmor angular velocity of 75.47 MHz lies between 1 ns and 3 ns. We can see that the correlation times of the C–H vectors are of the same order of magnitude as the correlation times of the interproton vectors. These measurements complete the analysis of the flexibility of the peptide. For example it is found from ^1H NOESY experiments that the $H_\alpha - H_\beta$ distance of residue 7 is fluctuating. This is confirmed by the ^{13}C experiments since the $C_\alpha H$ vector has a correlation time of the order of the whole molecule whereas the $H_\alpha - H_\beta$ vector has a 3-fold smaller correlation time as determined with NOESY. This means that the $NH - C_\alpha H - CO$ group is very rigid while the side chain rotates about the $C_\alpha - C_\beta$ bond. Generally the flexibility zones observed in ^{13}C experiments correspond to the flexibility zones observed in ^1H experiments. The only exception is at the ring closure. The $NH - H_\beta$ vector of residue 1 belongs to a rigid zone and its correlation time is of the order of 3 ns indicating the lack of mobility of the $NH - C_\alpha H - C_\beta H$ group. In contrast, the $C_\beta H$ vector exhibits a correlation time of 1.8 ns suggesting its mobility about the $C_\alpha - C_\beta$ bond. One explanation for these contradictory observations would be a fast but limited motion of $C_\beta H$ about $C_\alpha - C_\beta$, which leaves the $NH - C_\alpha H$ distance approximately constant.

Finally in this study we obtained interproton distance information from NOESY which can be divided into three sets. In the first set the distances are well-defined. In the second set it is impossible, owing to internal flexibility of the molecule, to extract accurate distances. The distances obtained using the crude $1/r^6$ approximation are likely to be wrong. Of course the existence of NOESY cross peaks indicates a proximity between protons, at least during part of the time. This second set contains 11 pairs of protons. For the last group of 48 NOESY connectivities it is more difficult to estimate the reliability of the distances determined from the $1/r^6$ law, owing to the weak values of the cross peak intensities. Some of the distances are certainly correct and others are not. One has to keep in mind that the weakness of the nOe signal does not necessarily imply a long interproton distance but may also reflect internal motion (Genest 1989).

All the above information will be used in a study of the three-dimensional structure of stendomycin by computer modeling using molecular mechanics. For this purpose, the nOe intensities will not all be used in a similar way in the refinement of the structure, they will be weighted depending on which set they belong to (Stawarz et al., manuscript in preparation). A detailed conformational analysis of stendomycin will be given and discussed elsewhere. It will be shown that important differences exist between our study and the originally model proposed by Pitner and Urry (1972).

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